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Akio Hizuta*

*Okayama University,

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CONCOMITANT PRESENCE OF ANTI-TUMOR EFFECTOR CELLS AND SUPPRESSOR CELLS IN THE SPLEEN OF TUMOR-BEARING MICE : THE NATURE OF SUPPRESSOR CELLS

Akio HIZUTA

Department of Surgery, Okayama University Medical School, Okayama 700, Japan
(Director : Prof. K. Orita)

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Abstract. In order to investigate the immunological responsiveness of tumor-bearing hosts to tumor cells, splenic suppressor cells from Ehrlich tumor-bearing mice that inhibited anti-tumor effector cell activity were characterized. *In vitro* cell-mediated cytotoxicity and cytostasis assays were performed to test for the existence of anti-tumor immunity. Suppressive activity assayed by cell mixture experiments became apparent with decline of anti-tumor immunity and progressive tumor growth. The cells mediating the suppression were found to be nylon wool column adherent T cells and inhibited T cell dependent cytotoxicity rather than non-T cell dependent cytostasis. *In vivo* cell transfer experiments demonstrated that intravenous injection of suppressor cells to a host already inoculated with tumor cells mixed with antitumor effector cells resulted in significant enhancement of tumor growth. This inhibition of *in vivo* neutralization assay by suppressor cells was found in not only allogeneic but also syngeneic tumor system. Splenectomy at the time of tumor resection endowed the host with stronger resistance against subsequent reinoculated tumor than sham-splenectomy did, reflected by prolonged survival times. These results suggest that splenectomy combined with surgical removal of the tumor is a useful treatment of clinical malignancies.

Key words : suppressor T cell, nylon wool column fractionation, tumor enhancement, splenectomy, tumor-bearing mice.

In tumor-bearing hosts (TBH), immunogenic tumors have been known to grow progressively and kill their hosts in spite of the development of immune responses against tumor antigens both in clinical neoplasia and in experimental animal models (1,2). The immune responses generated during early tumor growth eventually decline and often disappear as the tumors grow. The ineffectiveness of these immune responses in TBH has been explained by development of blocking factors such as antigens (3), antibodies (4), antigen-antibody complexes (5), or other soluble factors (6) in the serum of TBH, these blocking factors inhibiting the destruction of tumor cells by anti-tumor effector cells.

Recently, observations in human (7) and animal models (8-10) have indicated that the apparent unresponsiveness of TBH against tumor antigens may

be due to the concomitant presence of cells that possess the ability of suppressing the immune responses against tumor antigens through interaction with immunocompetenttcells rather than to the lack of immune responses in the host.

The present investigation was undertaken to characterize the cells that suppress the effector limb of cell-mediated immune responses in TBH against tumor cells. The *in vivo* effects of cell transfer and splenectomy on tumor growth were also examined.

MATERIALS AND METHODS

Mice. Male and female inbred strains of A/Ok and C3H/He mice were obtained from the Mouse Colony of the Okayama University Medical School. They were fed on Solid Feed MF (Oriental Yeast Co.) and tap water *ad libitum* throughout the course of the experiments. All mice used were 6 to 12 weeks of age.

Tumors. Non-strain specific Ehrlich tumor was obtained from the Section of Pathology, Okayama University Cancer Institute, and maintained *in vivo* by serial intraperitoneal (i.p.) passages in A/Ok and *in vitro* by culture in RPMI 1640 medium containing 10% fetal calf serum (FCS) (11). For studies of *in vitro* interaction between tumor cells and spleen cells, the culture cell line was used as target cell. MH-134 ascites hepatoma of C3H (12) was supplied by Dr. G. Fujii of the Institute of Medical Science, University of Tokyo, and maintained by serial i.p. passages in syngeneic hosts.

Tumor cell inoculation. A/Ok and C3H/He mice received subcutaneously (s.c.) 5×10^6 Ehrlih ascites tumor cells and 1×10^6 MH-134 cells respectively in the center of the back unless otherwise mentioned, and were used as tumor-bearing hosts. As the tumors enlarged, they were measured in 2 diameters with a vernier caliper.

Culture medium. The medium used was Roswel Park Memorial Institute 1640 medium (RPMI 1640) (Nissui Seiyaku Co. Ltd.) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin (Meiji Seika Co. Ltd.), 25 mM N'-2-hydroxy ethylpiperazine N'-2-ethane sulfonic acid (HEPES) buffer solution (Sigma Chemical Co., USA), and 20% FCS (Grand Island Biological Co., USA).

Spleen cell suspensions. Mice were sacrificed by exsanguination and the spleens were removed and placed into RPMI medium. Single cell suspensions were prepared by mincing, expulsing through a 5 ml syringe with a 18-gauge needle, and passing through a 150 mesh stainless steel screen. The cell suspensions were centrifuged at 180 x g for 5 min and erythrocytes were lysed with 0.83% Tris ammonium chloride solution. The cells were washed twice and counted with a hemocytometer, then diluted to appropriate concentrations with the culture medium. Viability was determined by 0.1% trypan blue dye exclusion tests.

Cytotoxicity assay. Visual microcytotoxicity assays were performed according to the method described by Takasugi and Klein (13). One drop of tumor cell suspension at a concentration of 1×10^4/ml was dispensed into each well of a Falcon 3034 microplate (Falcon Plastics, USA) with a 27-gauge needle attached to a tuberculin syringe. The plates were incubated for 4 h at 37°C in a humidified 5 % CO_2 incubator to allow cells to adhere. After incubation the plates were inverted for 20 min under incubation conditions and the medium was gently removed by aspiration. One drop of effector cell suspension at a concentration of 1×10^6/ml was added to the well. The plates were incubated for 48 h. At the end of the incubation, the plates were inverted for 20 min and the medium con-

taining effector cells and dead tumor cells were removed by aspiration. The plates were washed gently with phosphate-buffered saline (PBS). Living cells attached to the floor of the well were fixed with 1.25 % glutaraldehyde and stained with Giemsa. After cells were washed under a running tap water and dried, the residual target cells were counted visually under a microscope. The percent reduction was calculated by the following formula:

$$\% \text{ Reduction} = \left(1 - \frac{\text{number of cells in experimental cultures}}{\text{number of cells in control cultures}}\right) \times 100$$

Normal spleen cells were used in control cultures.

Cytostasis assay. The cytostasis assays were performed according to a modification of the test described by Chia and Festenstein (14). Five x 10^3 target cells and 2 x 10^5 effector cells were mixed and incubated at 37°C in a humidified 5% CO_2 incubator for 48 h in a final volume of 0.2 ml culture medium in the wells of round bottom microtiter plates (Cooke Engineering Co., USA). The cells were pulsed for the last 4 h of the incubation period with 1.0 μCi/well of ^{3}H-thymidine (specific activity 5.0 Ci/mM ; Radiochemical Centre, England) and harvested with a multiple cell-culture harvester (Skatron AS, Norway) onto glass fiber filters. The filters were then dried and transferred to glass scintillation vials. All samples were counted in 5 ml toluene containing 5.0 g/l of 2,5-diphenyloxazole (PPO) and 0.1 g/l of 1,4-bis(2-(5-phenyloxazolyl))-benzene (POPOP) (Packard Instrument Co., USA) on a Aloca scintillation spectrometer. All cultures were run in triplicate. The results were expressed as mean cpm ± standard error (S.E.) or percent reduction calculated by the following formula :

$$\% \text{ Reduction} = \left(1 - \frac{\text{cpm of experimental cells + target cells}}{\text{cpm of normal spleen cells + target cells}}\right) \times 100$$

Nylon wool column fractionation. Spleen cells were fractionated by nylon wool column passage with minimal modification of the method described by Julius *et al.* (15) and by Handwerger and Schwartz (16). About 0.6 g of nylon wool (Wako Junyaku Kogyo) was packed into the barrel of a 12 ml disposable plastic syringe (Top Surgical Mfg. Co. Ltd.) up to the 6 ml mark, autoclaved, and stored at room temperature until use. Before cell application, the nylon in the column was saturated and rinsed with the culture medium, and the column was incubated for 1 h at 37°C. After this preincubation, 1.0 - 1.5 x 10^8 spleen cells in 2ml of the medium preheated to 37°C were added to the column and allowed to flow into the nylon wool followed by 1 ml of 37° C medium. The column was then incubated for 45 min at 37°C. After this incubation, nonadherent cells were removed by adding the medium at 37°C to the column, and the rate of outflow was adjusted with a stopcock to approximately 1 ml/min until 10 ml of effluent was collected. The column was then washed with 100 ml of 37°C medium flowing rapidly through the column and this wash was discarded. Column-adherent cells were removed by compressing the nylon wool with the syringe plunger until most of the retained medium was expressed. The nylon wool was then teased with forceps, resatruated with 37°C medium, and the column was again compressed. This process was repeated until a total of 25 ml of medium had been collected. The nonadherent and adherent cells were pelleted, resuspended, and counted. Viability was assessed by trypan blue dye exclusion.

Petri dish adherent cell preparation. Ten ml of the spleen cell suspensions at a concentration of 1 x 10^6/ml were seeded in 10 cm plastic Petri dish (Falcon Plastics, USA) and in-

cubated for 45 min at 37°C. At the end of the incubation, nonadherent and loosely adhered cells were removed by vigorous agitation and the dishes were thoroughly washed five times with 20 ml of the culture medium. Adherent cells were then recovered from the dishes by scraping with a rubber policeman, washed, and resuspended in the culture medium.

Anti-Thy 1.2 (anti-θ) serum treatment. AKR anti-Thy 1.2 serum against C3H/He thymus cells (Searle Diagnostic, England) was used in detecting thymus-derived lymphocytes (T cells) in a complement-dependent cytotoxicity test. Spleen cells were incubated with a 1:20 final dilution of the serum for 30 min at 4°C, washed, and resuspended in 1:4 diluted guinea pig complement (C')(Kyokuto Seiyaku Industry Co. Ltd.) for 30 min at 37°C. Control spleen cells were treated with C' alone. The surviving cells were then washed twice and viable cells were counted by trypan blue dye exclusion.

In vivo tumor neutralization assay (Winn assay). Winn assay (17) was employed to test for anti-tumor reactivity of spleen cells *in vivo*. Mixtures of 1×10^7 spleen cells and 1×10^5 tumor cells in 0.2 ml Hanks' solution were incubated at 37°C for 30 min and injected s.c. into the center of the back of normal recipients. Tumor growth was monitored by serial measurement of tumor diameters with a vernier caliper.

Splenectomy. Under ether anesthesia, the peritoneal cavity was opened by a small incision under the left costal margin. The splenic pedicle was ligated with silk, the spleen was removed, and the abdominal wall was closed with silk. Sham-splenectomy was performed in the asme manner except that the spleen was not removed but left intact.

Statistics. Student's t test was used to evaluate the differences observed between mean values for groups. Differences were cosidered to be significant when probability (P) values <0.05 were obtained.

RESULTS

Appearance of anti-tumor activity after tumor inoculation. After 5×10^6 Ehrlich ascites tumor cells were inoculated s.c., serial cell-mediated immune reactivity of the host spleen cells against the tumor was tested by cytotoxicity and cytostasis assays (Fig. 1). The cytotoxicity reached a maximum at 10-15 days after tumor inoculation, and then diminished rapidly as the tumor grew. A minimal cytotoxic activity was detected after day 25. The cytostatic activity reached a maximum at around 15 days after tumor inoculation and decreased gradually thereafter. The decline of the cytostasis in the late course of tumor growth was less striking than that of the cytotoxicity.

Effect of anti-Thy 1.2 serum treatment of host spleen cells on cytotoxicity and cytostasis. In order to characterize the effector cells involved in cytotoxicity and cytostasis against tumor, spleen cells from 2 and 4 weeks TBH were treated with anti-Thy 1.2 serum and C' (Table 1). A representative experiment demonstrated that the treatment completely eliminated the cytotoxic activity of 2 weeks TBH spleen cells whereas it resulted in slight but significant decrease in cytostasis. The cytotoxic activity of 4 weeks TBH spleen cells was minimal and not affected by the treatment. The cytostatic activity of 4 weeks TBH spleen cells was significant and was also unaffected by the treatment. The results showed that T cells were predominantly responsible for cytotoxicity and cytostasis was mediated mainly

by non-T effector cells and partially by T effector cells. It was also indicated that T cell-mediated immune responses, both in cytotoxicity and in cytostasis, disappeared in the late course of tumor growth.

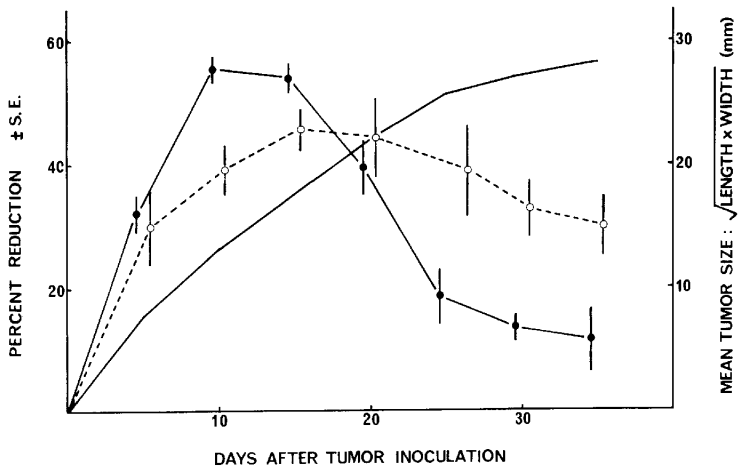

Fig. 1. Kinetics of tumor growth (——) and cytotoxic (●—●) and cytostatic (○—○) activities of spleen cells from TBH. A/Ok mice received 5 x 10^6 Ehrlich ascites tumor cells s.c. in the center of the back on day O. Spleen cells from TBH were prepared at timed intervals after tumor inoculation and tested for cytotoxicity and cytostasis against the tumor cells.

TABLE 1. ANTI-THY 1.2 SERUM TREATMENT OF SPLEEN CELLS FROM TBH

Effector cells	Cytostasis assay		Cytotoxicity assay
	cpm±S.E.	% Reduction	% Reduction
2 week spleen cells treated by			
None	18,735± 356	58.1	33.4
C' alone	19,128± 795	57.2	25.2
Anti-Thy 1.2 plus C'	24,458± 692*	45.2*	2.9*
4 week spleen cells treated by			
None	28,618± 853	35.9	9.5
C' alone	29,652±1,682	33.6	7.3
Anti-Thy 1.2 plus C'	34,507± 452	22.8	3.4
Normal spleen cells			
Untreated	44,668±1,836	Standard	Standard

*Significantly different from cells treated by C' alone, $P<0.05$.
TBH, tumor-bearing host.

Nylon wool column fractionation of the spleen cells from TBH in the late course. The possibility that the disappearance of T cell-mediated immune responses from advanced TBH spleens was due to a lack of T effector cells was examined. The spleen cells from 4 weeks TBH were filtered through a nylon wool column and nonadherent cells were separated from adherent cells. The fractionated cells and the unfractionated cells were then assayed for cytotoxicity and cytostasis against tumor cells. As shown in Table 2, column-nonadherent cells demonstrated a strong cytotoxicity whereas the unfractionated cells and column-adherent cells were not cytotoxic. The results indicated that the decreased cytotoxicity of spleen cells from advanced TBH was not due to a lack of effector T cells but to either the presence of suppressor cells in the spleen or to a simple dilution of effector T cells by nonreactive cells.

TABLE 2. NYLON WOOL COLUMN FRACTIONATION OF SPLEEN CELLS FROM 4 WEEK TBH

Effector cells	Cytostasis assay		Cytotoxicity assay
	cpm ±S.E.	% Reduction	% Reduction
4 week spleen cells			
Unfractionated	28,516±1,699	40.6	2.4
Adherent	27,854± 852	42.0	-3.4
Nonadherent	34,317±3,611	28.5	41.1*
Normal spleen cells			
Unfractionated	48.004±3,347	Standard	Standard

*Significantly different from unfractionated cells, $P<0.05$.
TBH: see Table 1.

Active suppression of cytotoxicity in cell-mixture experiments. Since spleen cells from 2 weeks TBH showed a significant level of cytotoxicity and cytostasis against the target tumor cells, they were mixed with spleen cells obtained at various times after tumor inoculation at a ratio of 1:1 and the mixtures were tested for cytotoxicity and cytostasis in order to detect the suppressive effect. As shown in Fig. 2, the cytotoxic activity was strongly suppressed by the addition of spleen cells from TBH in the late course of the tumor growth, whereas the added spleen cells from TBH were not suppressive in cytostasis assay through the entire period of the tumor growth.

Suppressive activity of nylon wool column fractionated spleen cells. To examine the suppressive activity of nylon wool column fractionated spleen cells, spleen cells from 4 weeks TBH were fractionated by the column and mixed with 2 weeks TBH spleen cells. The mixtures were then assayed for cytotoxicity and cytostasis. From the data listed in Table 3, it was evident that although cytostasis was not affected by either fraction, cytotoxicity was suppressed by unfractionated cells and cells of the column-adherent fraction, indicating that the suppression of cytotox-

icity was mediated by a population of nylon wool column adherent cells.

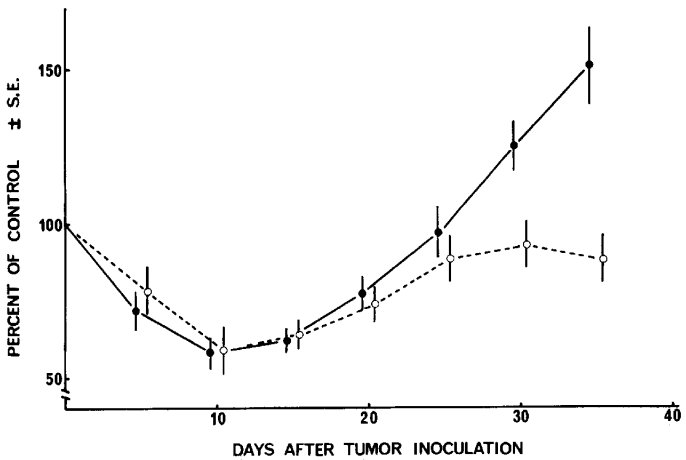

Fig. 2. Effects of spleen cells from TBH on anti-tumor effector cell activity. Spleen cells from 2 weeks TBH were used as anti-tumor effector cells. Spleen cells from TBH at timed intervals after inoculation of 5 x 10^6 Ehrlich ascites tumor cells were mixed with the effector cells at a ratio of 1:1, then tested for cytotoxicity (●—●) and cytostasis (○—○). The resultant target cell number in cytotoxicity assay or cpm in cytostasis assay was compared with that of normal mouse spleen cells which were mixed with the effector cells at a ratio of 1:1.

TABLE 3. SUPPRESSIVE ACTIVITY OF NYLON WOOL COLUMN FRACTIONATED SPLEEN CELLS

Effector cells	Cytostasis assay		Cytotoxicity assay
Cells added to 2 week cells[a]	cpm ±S.E.	% Reduction	% Reduction
Tumor-bearing spleen cells[b]			
Unfractionated	20,860± 555	56.5	5.2*
Adherent	26,928±1,717	43.9	3.1*
Nonadherent	34,766±1,413	27.6	34.2
Normal spleen cells			
Unfractionated	28,355± 520	40.9	36.6
Adherent	28,715± 752	40.2	22.0
Nonadherent	34,340± 868	28.5	30.5

a Cells were added to spleen cells from 2 week TBH at the ratio of 1:1.

b Spleen cells from 4 week TBH.

* Significantly different from normal spleen cells, $p<0.05$.

TBH, see Table 1.

Effect of anti-Thy 1.2 serum treatment on the suppressive activity. In order to test whether the suppression was mediated by T cells, spleen cells from 4 weeks TBH

were treated with anti-Thy 1.2 serum and C' and tested for suppressive activity. As shown in Table 4, the suppression of cytotoxicity was completely abrogated by the treatment. The results indicated T cell dependency of the suppression.

TABLE 4. EFFECT OF ANTI-THY 1.2 SERUM TREATMENT ON SUPPRESSIVE ACTIVITY

Effector cells	Cytostasis assay		Cytotoxicity assay
Cells added to 2 week cells[a]	cpm±S.E.	% Reduction	% Reduction
Tumor-bearing spleen cells[b] treated by			
None	18,715±1,260	58.1	5.2
C' alone	20,027± 979	55.2	3.2
Anti-Thy 1.2 plus C'	26,738±2,035*	40.2*	25.9*
Normal spleen cells			
Untreated	29,592±1,193	33.8	31.0

a Cells were added to spleen cells from 2 week TBH at the ratio of 1:1.
b Spleen cells from 4 week TBH.
*Significantly different from cells treated by C' alone, $P<0.05$.
TBH, see Teble 1.

Effect of Petri dish adherent cells. In order to examine the involvement of Petri dish adherent cells in the suppression, the adherent cells prepared from 4 weeks TBH and normal spleen cells were mixed with 2 weeks TBH spleen cells and the mixtures were assayed for cytotoxicity and cytostasis. As shown in Table 5, there was no difference between advxnced TBH and normal Petri dish adherent cells in the effect on cytotoxicity and cytostasis assays, indicating that macrophages were not responsible for the suppression.

TABLE 5. EFFECT OF PETRI DISH ADHERENT CELLS

Effector cells	Cytostasis assay		Cytotoxicity assay
Cells added to 2 week cells[a]	cpm±S.E.	% Reduction	% Reduction
Petri dish adherent cells from			
4 week TBH	25,208±596	47.5	27.1
Normal mouse	27,169±999	43.4	28.6

a Cells were added to spleen cells from 2 week TBH at the ratio of 1:1.
TBH, see Table 1.

Effect of spleen cells from advanced TBH on in vivo tumor neutralization assay. Attempts to examine whether the suppressor cells detected *in vitro* functioned *in vivo* were made. Five x 10^7 spleen cells from 4 weeks TBH were injected intravenously (i.v.) four times at intervals of one week into A/Ok mice which received s.c. 1 x 10^5 Ehrlich ascites tumor cells mixed with 1 x 10^7 spleen cells from 2 weeks TBH.

The resultant tumor growth was found to be significantly enhanced in mice which received 4 weeks TBH spleen cells, as compared with mice which received normal spleen cells (Fig. 3). A similar experiment was performed in a syngeneic tumor system. Because of lower antigenic activity of the syngeneic MH-134 tumor than that of allogeneic Ehrlich tumor, immune spleen cells were used as anti-tumor effector cells instead of TBH spleen cells. For immunization, 1 x 10^6 MH-134 tumor cells were inoculated in the back of C3H/He mice and the tumor was completely resected surgically one week later. This procedure was repeated three times at intervals of 2 weeks. One x 10^5 MH-134 cells were mixed with 5 x 10^6 immune cells and 5 x 10^6 spleen cells from 4 weeks TBH or normal spleen cells, and the mixtures were inoculated s.c. in the center of the back of normal C3H/He mice after preincubation. As is clearly shown by the tumor growth curves in Fig. 4, spleen cells from 4 weeks TBH suppressed the effect of immune cells and enhanced the tumor growth significantly. These results suggested that cells which suppressed the *in vivo* function of anti-tumor effector cells were present in the spleen of advanced TBH.

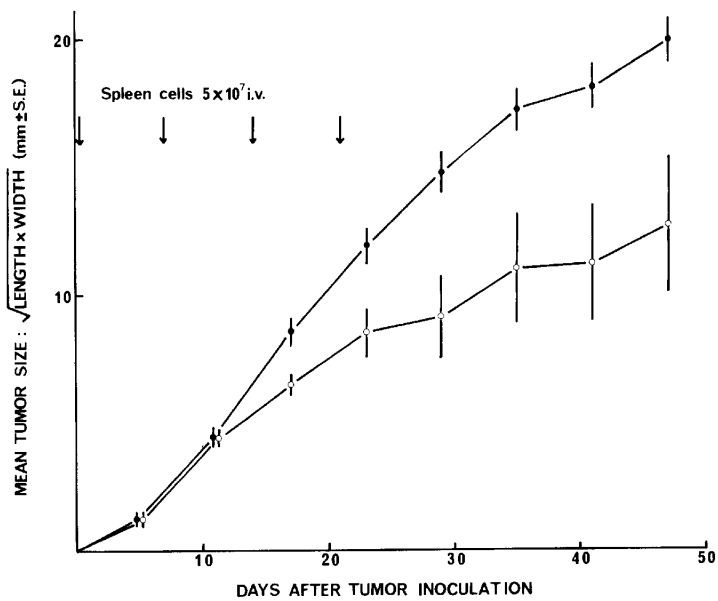

Fig. 3. Tumor enhancing effect of spleen cells from TBH. Mixtures of 1 x 10^5 Ehrlich ascites tumor cells and 1 x 10^7 anti-tumor effector cells were inoculated s.c. into the back of A/Ok mice on day O. Spleen cells from 2 weeks TBH were used as anti-tumor effector cells. Five x 10^7 spleen cells from 4 weeks TBH (●—●) or normal mice (○—○) were injected i.v. on day 0,7,14, and 21. Each group consisted of 15 mice.

Effect of splenectomy. To determine whether removal of suppressor cells residing in the spleen of TBH cotributed to host resistance against the tumor, splenectomy combined with tumor resection was performed. A week after inoculation of 1 x 10^6, Ehrlich ascites tumor cells, A/Ok mice were splenectomized and the tumor was resected. At the same time a small dose, 1 x 10^6, of the tumor cells was reinoculated and the mice were inspected for survival. Sham-splenectomy combined tumor resection was used as a control. Although there was no significant difference in tumor size between splenectomized and sham-splenectomized mice, significant prolongation of survival was observed in splenectomized mice (Fig. 5). The mean survival time was 42.9 ± 3.2 (± S.E.) days in splenectomized mice, as compared with 33.4 ± 2.1 days in sham-splenectomized mice ($P<0.05$). These results indicated that splenectomy done at the time of tumor resection led the host to be more resistant to the tumor than did tumor resection alone.

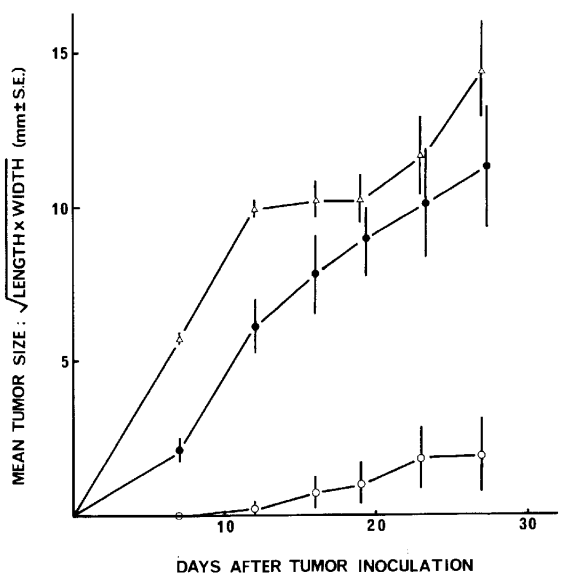

Fig. 4. Suppressive effect of spleen cells from TBH on *in vivo* tumor neutralization assay in syngeneic tumor system. C3H/He mice received 1 x 10^5 MH-134 cells mixed with 1 x 10^7 normal C3H/He spleen cells (Δ—Δ), 5 x 10^6 immune cells plus 5 x 10^6 normal mouse spleen cells (○—○), or 5 x 10^6 immune cells plus 5 x 10^6 spleen cells from 4 weeks MH-134 tumor-bearing syngeneic hosts (●—●). Each group consisted of 15 mice.

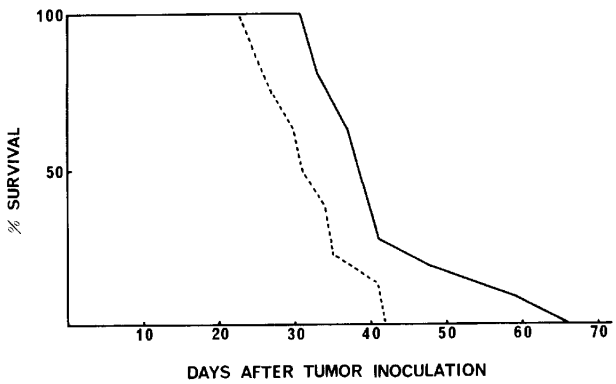

Fig. 5. Effect of splenectomy combined with tumor resection on resistance to the tumor growth. Splenectomy (———, 11 mice) or sham-splenectomy (-------, 8 mice) combined with tumor resection was performed 7 days after inoculation of 5 x 10^6 Ehrlich ascites tumor cells in A/Ok mice. On the day of the operation, 1 x 10^6 tumor cells were reinoculated s.c. in the back and survival was monitored.

DISCUSSION

There is a vast body of evidence demonstrating that different types of immunocompetent cells or functionally distinct subsets of cells within the same type play an important role in the negative regulation of cell-mediated immune responses, such as graft-versus-host reaction (18), contact sensitivity (19), mixed lymphoctye reactiion (MLR)(20), and *in vitro* induction of cytotoxic lymphocytes (21,22). Consequently, one may postulate the existence of suppressor cells that inhibit cell-mediated immune responses in TBH and cause progressive growth of immunogenic tumors. It is necessary, therefore, to understand the nature of these cells and explore experimental regimens in which depletion of these cells can be achieved to faver the development of effective anti-tumor reactions.

The results of this paper demonstrate the concomitant presence of anti-tumor cytostatic and cytotoxic effector cells and a cell population which inhibited the activity of these anti-tumor cytotoxic effector T cells in the spleen of highly immunogenic Ehrlich tumor (23) -bearing mice. When the spleen cells from advanced TBH which were apparently not cytotoxic to the tumor cells were fractionated with nylon wool column, the column-adherent cell population was able to suppress the activity of cytotoxic effector T cells. The column-nonadherent cell population was not suppressive but cytotoxic to the tumor cells. The suppressive activity was abrogated by treatment with anti-Thy 1.2 serum and C'. These results suggest that a subpopulation of T cells which can be separated in the nylon wool column adherent fraction of spleen cells from advanced TBH is responsible for the suppression of cytotoxicity and the induction of an apparent lack of cytotoxicity against tumor.

These results are in agreement with those of Yu *et al.* in humans (7) and Schaaf-Lafontaine in mice (10) demonstrating that adherent T cells from TBH suppress anti-tumor cytotoxic activities at the effector stage *in vitro*. Adherent T cells which suppress the generation of cytotoxic T cells have been found in different experiments. Hodes and Hathcock (22) have demonstrated that nylon wool column adherent T cells in normal mouse spleen cells suppress *in vitro* induction of cytotoxic T lymphocytes against allogeneic spleen cells, trinitrophenyl (TNP) -modified syngeneic spleen cells, or syngeneic tumor cells. Holán *et al.* (24) have attributed *in vitro* unresponsiveness in neonatal transplantation tolerance to specific nylon wool column adherent suppressor cells which inhibit the activity of potentially reactive cells to react to the tolerated antigens.

It is possible that a different population of spleen cells is involved in the suppression demonstrated in our study. It cannot be ruled out that the collaboration of nylon wool column adherent non-T cells (e.g. macrophages) and nonadherent T cells, or adherent and nonadherent T cells might be required for the expression of the suppressor activity. Macrophages isolated from TBH have been shown to be implicated in the suppression of T cell reaction by a number of workers (25,26). However, the data are not consistent with our data which show that a subpopulation of Thy-1 positive cells was able to express suppressor cell activity. Folch and Waksman (20) reported glass wool column adherent T cells in normal rat spleens which suppress MLR, and hypothesized a suppressor T cell-macrophage complex as a regulatory unit (27). Recently, Tada *et al.* (28) showed that two types of T cells, adherent and nonadherent to nylon wool, were required for the induction of effective suppression in humoral immunity. The Ly phenotype of the adherent T cells has been shown to be $Ly123^+$ (29). Their results suggest that $Ly23^+$ cells may stimulate $Ly123^+$ cells to become suppressive under certain situations. It has been also shown that $Ly123^+$ Suppressor effector cells are induced by signals from antigen-stimulated Lyl^+ cells in a regulatory circuit termed feedback inhibition (30). These results are of great relevance to the present observation that the generation of supressor T cells appeared to be preceded by the generation of positive T cell-mediated immunity against tumor.

In the light of reported discrepancies between results of *in vitro* assay and *in vivo* responses in tumor immunity (31), *in vivo* tumor neutralization assay was employed in the present study. Spleen cells which inhibited *in vitro* cytotoxicity, rather than cytostasis, were able to enhance *in vivo* tumor growth when they were transferred with tumor cells and anti-tumor effector cells into normal recipients. This result suggests that the suppressor cells inhibit *in vivo* activity of cytotoxic T cells.

Although it is not certain from the present study whether the mechanism of suppression is specific or nonspecific, *in vivo* suppression observed in syngeneic tumor system suggests that the invlovement of suppressor cells in tumor immunity is not an exclusive phenomenon of allogeneic system. It can be postulated that

specific or nonspecific activation of T cell-mediated immune responses *in vivo* would have bidirectional effects on tumor growth, inhibition or enhancement. Therefore, it is of importance to mention that attempts at immunotherapy of malignancies in humans as well as in animals must be made with caution.

Splenectomy was carried out in order to remove suppressor cells in the spleen of TBH. The results demonstrated that splenectomy combined with tumor resection rendered the host more resistant against tumor growth than sham-splenectomy plus tumor resection. The effect of splenectomy on tumor growth gave conflicting results. Some workers have reported splenectomy to result in retardation of tumor growth (8,32), no effect (33), or facilitation (34). It has also been reported that the effect of splenectomy varied according to the tumor load (35), or to the timing of the operation relative to tumor inoculation (36, 37). Although many reports dealt with the effect of splenectomy performed before the inoculation of tumor cells, the clinical implication is that splenectomy after the establishment of a tumor may augment the host's immune responses against the tumor. In this respect, the results of the present study suggest that splenectomy combined with tumor resection is an available therapeutic aid to protect a host from recurrence or metastasis of the tumor.

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